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Chromatin and DNA Replication

David M. MacAlpine[1] and Geneviève Almouzni[2]

[1]Department of Pharmacology and Cancer Biology, Duke University, Durham, North Carolina 27710
[2]Institut Curie, Section de Recherche, UMR218 du CNRS, 75231 Paris Cedex 05, France
Correspondence: david.macalpine@duke.edu

The size of a eukaryotic genome presents a unique challenge to the cell: package and organize the DNA to fit within the confines of the nucleus while at the same time ensuring sufficient dynamics to allow access to specific sequences and features such as genes and regulatory elements. This is achieved via the dynamic nucleoprotein organization of eukaryotic DNA into chromatin. The basic unit of chromatin, the nucleosome, comprises a core particle with 147 bp of DNA wrapped 1.7 times around an octamer of histones. The nucleosome is a highly versatile and modular structure, both in its composition, with the existence of various histone variants, and through the addition of a series of posttranslational modifications on the histones. This versatility allows for both short-term regulatory responses to external signaling, as well as the long-term and multigenerational definition of large functional chromosomal domains within the nucleus, such as the centromere. Chromatin organization and its dynamics participate in essentially all DNA-templated processes, including transcription, replication, recombination, and repair. Here we will focus mainly on nucleosomal organization and describe the pathways and mechanisms that contribute to assembly of this organization and the role of chromatin in regulating the DNA replication program.

HISTONE VARIANTS AND MODIFICATIONS

The histone octamer consists of two molecules of each of the core histones H3, H4, H2A, and H2B. The H3 and H4 histones form a tetramer $(H3-H4)_2$, that organizes the central 70 bp of the DNA for the further addition of the two flanking H2A–H2B dimers (Luger et al. 1997). The constituent core histones (H2A, H2B, H3, and H4) represent the bulk of nucleosome-associated histones. However, multiple histone variants contribute to the diversity of chromatin structure and function (Ahmad and Henikoff 2002a; Kamakaka and Biggins 2005; Probst et al. 2009). Sequence variants are found in metazoans for all of the histones, except for histone H4. Distinct assembly mechanisms exist for the different variants, allowing them to be deposited in a replication-dependent or -independent manner (see below), and may contribute to their role in defining epigenetic states.

H3 Variants

Centromere-specific H3 variants are found in all eukaryotes and are referred to as CenH3s. The mammalian centromere-associated protein (CENP-A) is an essential protein that defines the location and function of centromeres

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(Stimpson and Sullivan 2010). The various CenH3s have a conserved globular domain but feature unique amino-terminal tails that are important for kinetochore function. The rapid evolution of centromeric repeats coupled with the adaptive evolution of CenH3 (Henikoff et al. 2001) underscores the importance of variant histones in maintaining epigenetic identity and state.

All metazoans possess a replication-dependent histone variant corresponding to H3.2 in mammals. Additional replicative variants are also present in mammals, such as H3.1, which differs from H3.2 by a single amino acid. The expression of H3.1 and H3.2 is tightly coupled to the cell cycle, showing a peak of synthesis during S phase and providing the major source of histones for deposition behind the replication fork.

H3.3 is a replacement variant that differs from H3.2 by four amino acids and is enriched in specific regions of the genome. Constitutively expressed throughout the cell cycle and in quiescent cells, it is deposited onto DNA in a replication-independent manner (Ahmad and Henikoff 2002b; Tagami et al. 2004; Ray-Gallet et al. 2011). The eviction of nucleosomes by transcription and chromatin remodeling events provides opportunities for deposition of newly synthesized H3.3. This can explain how H3.3 marks active and dynamic chromatin and accumulates in transcribed regions, enhancers, promoters (Mito et al. 2007), and origins of DNA replication (Deal et al. 2010; MacAlpine et al. 2010). This deposition depends primarily on a separate assembly pathway (ASF1/HIRA) discussed below.

H2A/H2B Variants

H2A.Z is the most conserved H2A variant across different species, even more so than the canonical H2A. H2A.Z is enriched at the flanks of nucleosome-depleted regions surrounding active promoters and promotes gene activation (Raisner and Madhani 2006). In *Saccharomyces cerevisiae*, H2A.Z acts as a barrier at promoters, preventing the spread of silent heterochromatin (Meneghini et al. 2003).

H2A.X represents 2%–25% of the mammalian H2A histone pool and is structurally similar to the other H2A variants except for a serine (Ser-139) positioned four amino acids from the carboxyl terminus (Kinner et al. 2008). Ser-139 is phosphorylated in response to DNA damage, resulting in the accumulation of phospho-H2A.X (also referred to as γ-H2A.X) in chromatin surrounding the DNA damage lesion. Subsequent repair activities are recruited to the lesion via interactions with γ-H2A.X (Stucki et al. 2005). Of note, *S. cerevisiae* has a single H2A mostly related to H2A.X, whereas *Drosophila* has a single bifunctional histone variant, H2A.v, that combines the properties of H2A.Z and H2A.X.

MacroH2A is a histone variant with a large carboxy-terminal ligand-binding domain called the macro domain (Pehrson and Fried 1992). MacroH2A is found enriched on the inactive X chromosome (Costanzi and Pehrson 1998). Conversely, H2A.BbD (Barr body deficient) is a divergent variant specific to vertebrates that is excluded from the inactive X chromosome and colocalizes with acetylated histone H4, suggesting a role in maintaining euchromatic function (Chadwick and Willard 2001).

Temporal Expression of Histones and Variants

In each cell cycle, a sufficient amount of core histones must be synthesized to provide ∼20 million new nucleosomes for packaging the newly replicated daughter strands. Not surprisingly, the synthesis of the canonical core histones is tightly coupled to the cell cycle (Marzluff and Duronio 2002; Gunjan et al. 2005). Both transcriptional and posttranscriptional regulatory controls exist to ensure sufficient histone levels and prevent the accumulation of excess histones. Interestingly, histone mRNAs lack a poly(A) tail and instead have a stem-loop structure at the 3′ end, which is important for stability and translation in mammals (Marzluff et al. 2008). Failure to regulate histone levels can have profound consequences on cell-cycle progression and genome stability. Insufficient histone levels can trigger a cell-cycle arrest in *S. cerevisiae* (Han et al. 1987; Kim et al. 1988)

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and impair S-phase progression in mammalian systems (Nelson et al. 2002). Similarly, excess histones are also harmful, resulting in DNA damage and genome instability (Gunjan and Verreault 2003).

Posttranslational Modifications

The abundance of lysine residues within the nonconserved histone tails and their ability to be posttranslationally modified provides for a massive combinatorial repertoire, denoted a "histone code," and has the potential to regulate many chromatin-templated functions (Jenuwein and Allis 2001). These regulatory functions potentially include transcription, replication, repair, recombination, and chromatin condensation and segregation. Posttranslational modification (PTM) of histones includes, but is not limited to, methylation, acetylation, SUMOylation, ubiquitination, and ribosylation of lysine residues and phosphorylation on serine and threonine residues (Kouzarides 2007). Notably, these modifications are reversible. Finally, several of these PTMs (and histone variants) can contribute to the specific marking of chromatin states, which in some cases can be stably propagated through multiple cell divisions; as such, they are believed to be purveyors of epigenetic information. Given the importance of not only the nucleosomal structure itself for genome functions but also the dynamic regulatory properties of chromatin, it is important to understand the mechanisms that govern the disassembly and reassembly of chromatin states following passage of the replication fork.

ASSEMBLY OF CHROMATIN

An immediate consequence of DNA replication is the disruption of the existing chromatin structure by passage of the replication fork. Two pathways contribute to the reassembly of chromatin on the nascent DNA. In the first, disruption of the DNA–histone octamer interaction ahead of the replication fork generates parental histones that can be recycled behind the fork, and the second pathway facilitates the deposition of newly synthesized histones onto nascent DNA (Fig. 1). The segregation of parental histones combined with the de novo deposition of newly synthesized histones are critical for genome stability and the inheritance of chromatin states.

Replication-Dependent Deposition of Histones

Our understanding of the mechanisms and factors involved in histone deposition began with pioneering cell-free systems enabling chromatin assembly in *Xenopus laevis* egg extracts (Laskey et al. 1977). Next, the in vitro replication system using SV40 origin-containing plasmids along with the SV40 large T antigen and human cytosolic extracts provided a complementation assay in which addition of nuclear extracts enabled their efficient assembly into minichromosomes (Stillman 1986). This assay led to the biochemical identification of human chromatin assembly factor 1 (CAF-1), a histone chaperone with the unique property of promoting deposition of histones H3–H4 onto replicating DNA (Smith and Stillman 1989), a function that is evolutionarily conserved. The CAF-1 complex comprises three subunits (p150, p60, and RbAp48 in mammals) (Kaufman et al. 1995; Verreault et al. 1996). CAF-1 is targeted to replication forks through an interaction with proliferating cell nuclear antigen (PCNA), a ring-shaped homotrimeric protein that serves as a processivity factor for the DNA polymerases (Shibahara and Stillman 1999; Moggs et al. 2000). This CAF-1–PCNA interaction depends on phosphorylation of the large subunit of CAF-1 (p150) by the replicative kinase Cdc7-Dbf4 in human cells (Gerard et al. 2006), which offers a potential means to ensure a tight coordination between histone deposition and ongoing DNA replication. The importance of CAF-1 in vivo is shown by loss-of-function studies leading to loss of viability during development in mouse (Houlard et al. 2006), *Xenopus* (Quivy et al. 2001), and *Drosophila* (Song et al. 2007; Klapholz et al. 2009) and impaired S-phase progression in human cells (Hoek and Stillman 2003).

Together with CAF-1, another H3–H4 histone chaperone, antisilencing function 1 (ASF1), identified initially in a yeast screen for silencing

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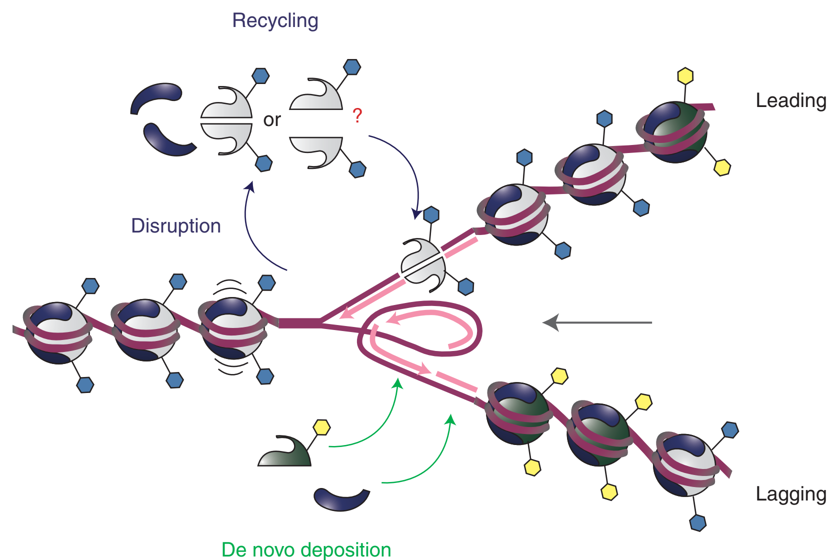

	Nucleosome		Fork movement
	Parental $(H3–H4)_2$		H2A–H2B
	Old H3.1–H4 dimer		Parental PTM
	New H3.1–H4 dimer		H4K5,K12ac
	New DNA		

Figure 1. Histone dynamics at the replication fork. New and parental histones are incorporated into chromatin behind the replication fork. The disassembly of nucleosomes ahead of the replication fork provides a parental pool of H3–H4 tetramers or dimers for assembly by histone chaperones. Newly synthesized dimers of H3–H4 histones are also deposited at the fork. The recycling of parental histones provides a means to maintain and propagate distinct chromatin states. H2A–H2B dimers are assembled into chromatin following the deposition of the H3–H4 tetramer.

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defects on overexpression (Le et al. 1997), facilitates chromatin assembly coupled to DNA synthesis in vitro (Tyler et al. 1999; Mello et al. 2002). However, the addition of purified ASF1 to human cell extracts or *X. laevis* egg extracts depleted for histone cell cycle regulation defective homolog A (HIRA), CAF-1, and ASF1 is not sufficient to promote histone deposition. This indicates that ASF1 is unlikely to play a direct role in either the replication-coupled or -independent chromatin assembly pathways (Mello et al. 2002; Ray-Gallet et al. 2007). Instead, ASF1 acts as a histone donor for the histone chaperone CAF-1 during DNA replication or repair, a collaboration conserved in various organisms. ASF1 interacts with the B-domain of the p60 subunit of CAF-1 (Tyler et al. 2001; Mello et al. 2002; Sanematsu et al. 2006; Tang et al. 2006; Malay et al. 2008) at a site opposite to that of its interaction with H3–H4 (English et al. 2006; Natsume et al. 2007). Formation of a ternary complex (CAF-1–ASF1–H3–H4) could thus function as an intermediate enabling histones to be handed over from one chaperone to the next. The transfer of histones from ASF1 to CAF-1 as part of an "assembly line" would ensure an efficient histone deposition coupled to DNA replication (Fig. 2).

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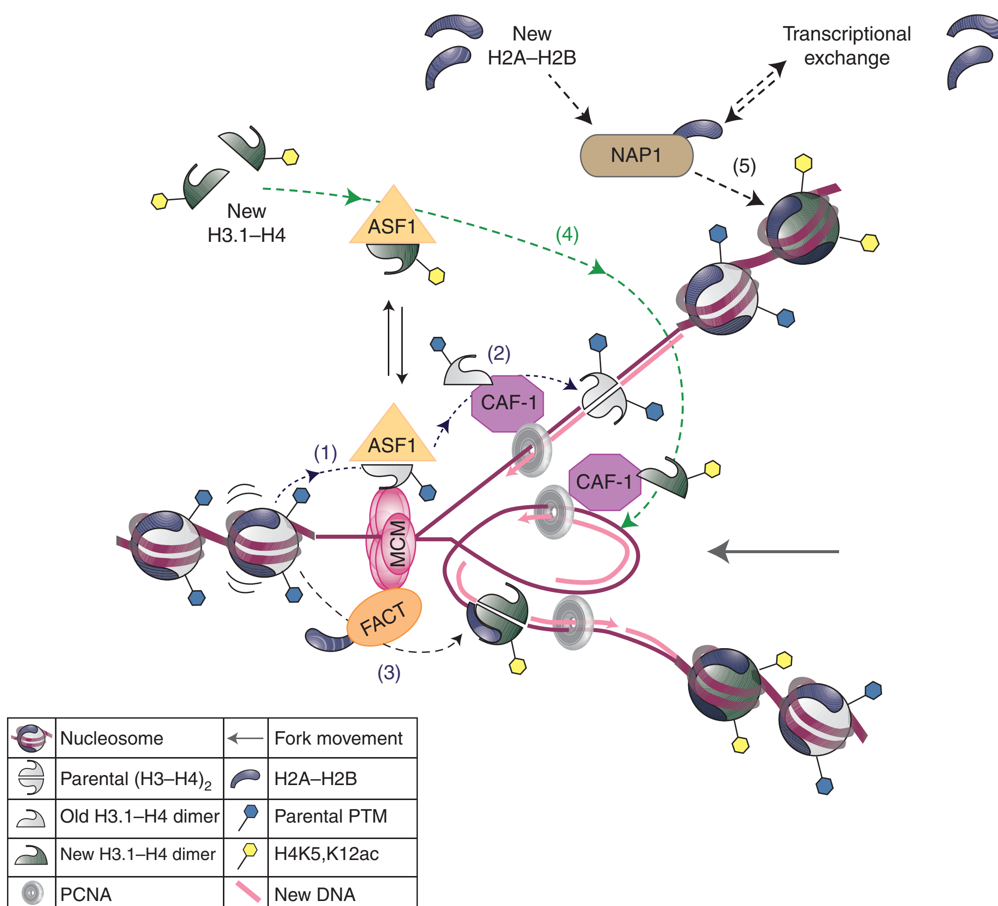

Figure 2. Chromatin assembly is mediated by a network of histone chaperones. Disruption of chromatin in front of the fork aided by ATP-dependent chromatin remodeling activities and the Mcm2–7 helicase results in release of parental histones. Interactions between the Mcm2–7 complex and the histone chaperones ASF1 and FACT may aid in the disassembly of the nucleosome and provide a means for sequestering parental histones at the fork. ASF1 may function in a histone chaperone "assembly line" to split $(H3–H4)_2$ tetramers into H3–H4 dimers (*1*) for deposition by CAF-1 on either the leading or lagging strand (*2*). CAF-1 is tethered to the leading and lagging strands via an interaction with PCNA, thereby providing a potential mechanism for semiconservative deposition of parental histones. Similarly, FACT would facilitate the retention and assembly of H2A–H2B dimers onto the nascent DNA (*3*). Newly synthesized histone H3–H4 dimers are delivered to the replication fork by ASF1 for deposition by CAF-1 (*4*) or, in the case of histone H2A–H2B dimers, by NAP1 (*5*).

To complete nucleosome formation following the delivery of two dimers of H3–H4 onto newly synthesized DNA, the subsequent addition of histones H2A–H2B involves the nucleosome assembly protein 1 (NAP1) chaperone (Zlatanova et al. 2007). Given that the FACT (facilitates chromatin transcription) complex also acts as an H2A–H2B chaperone in transcription, DNA replication, and DNA repair, it could perhaps help to provide a connection with NAP1 (Krogan et al. 2006). However, it should be noted that incorporation of new H2A–H2B does not necessarily have to be tightly linked to DNA replication, as significant H2A–H2B exchange also occurs outside replication (Kimura and Cook 2001).

Other histone chaperones should also be considered as players in chromatin assembly

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and histone dynamics; for example, nuclear autoantigenic sperm protein (NASP), initially reported as a histone H1 linker chaperone (Finn et al. 2008), was later found to function as an H3–H4 chaperone (Osakabe et al. 2010) as part of a multichaperone complex (Tagami et al. 2004; Groth et al. 2005). Recent work indicates that NASP may act to fine-tune soluble H3 levels by counteracting degradation involving the chaperone-mediated autophagy pathway (Cook et al. 2011).

Replication-Independent Deposition of Histone Variants H3.3 and CenH3

In contrast to the replicative histone variants H3.1 and H3.2, which are deposited throughout the genome during replication, the replacement variant H3.3 accumulates in actively transcribed chromatin regions, as first shown in *Drosophila* (Ahmad and Henikoff 2002b). H3.3 nucleosomes are enriched with "active" PTMs compared with the replicative variants (McKittrick et al. 2004; Hake et al. 2006; Loyola et al. 2006). The accumulation of histone H3.3 at promoters of active genes or at regulatory elements exploits a replication-independent mechanism involving the histone chaperone HIRA (Ray-Gallet et al. 2011). However, H3.3 is not only confined to sites of active transcription but is also enriched at other genomic regions depending on the developmental context. This is illustrated at the time of fertilization with a massive and global accumulation of H3.3 onto sperm DNA (Loppin et al. 2005), and in embryonic stem cells with the accumulation of H3.3 at telomeres (Goldberg et al. 2010; Wong et al. 2010). How these events are controlled and which factors are involved are beginning to be unraveled (for review, see Elsaesser et al. 2010). Recent data showing that HIRA can directly bind to DNA suggest a mechanism whereby the HIRA-dependent pathway could act as a gap-filling mechanism to restore nucleosomal organization wherever it may be compromised (Ray-Gallet et al. 2011), for example, as a result of faulty CAF-1 deposition. Thus, one should consider these pathways as interlinked to restore nucleosome density after fork passage. Other H3.3 chaperones have also been uncovered, and their exact role in H3.3 deposition may depend on the cell type and context (Elsaesser et al. 2010).

The mammalian CenH3, CENP-A, also called the deviant H3 (Wolffe and Pruss 1996), is highly divergent from H3 and stands out as the best example of a histone H3 variant that specifies a functional genomic locus. Specifically, CenH3 defines the centromere (Warburton et al. 1997), where it serves as an essential platform for kinetochore assembly (Allshire and Karpen 2008). During replication of centromeric chromatin, CenH3 nucleosomes become diluted to half the initial concentration on daughter chromatin (Shelby et al. 2000; Jansen et al. 2007). It is not until the next G_1 phase that new CenH3 gets incorporated again (Jansen et al. 2007; Schuh et al. 2007). This case illustrates a situation in which the disruption of chromatin during replication is clearly separated from the functional reassembly of the chromatin outside of S phase. With respect to the associated mechanism of the human CenH3 deposition, the recent identification of Holliday junction recognition protein (HJURP) is particularly enlightening. HJURP is a CenH3 chaperone, localized at centromeres precisely from late telophase to early G_1, which promotes the specific targeting/incorporation and maintenance of CenH3 at centromeres (Dunleavy et al. 2009; Foltz et al. 2009). So far, we have considered the situation from the point of view of how to restore the initial chromatin state after a disruptive event such as replication. However, the following alternative consideration is equally valid: Incorporation of CenH3 in G_1 may be programmed in anticipation of the disruptive event during replication, rather than being a restoration of half the pool of CENP-A. Whichever way one looks at this issue, it provides a general conceptual framework for the mechanism by which chromatin marks can be dealt with during the cell cycle.

DISRUPTION OF CHROMATIN AT THE REPLICATION FORK

Transcription and DNA replication require that the chromatin be disrupted ahead of the RNA or

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DNA polymerase complex. Although the replicative helicase, Mcm2–7 complex, can in theory provide the means to disrupt nucleosomes ahead of the replication fork, it is important to consider how distinct ATP-dependent chromatin remodeling complexes facilitate the sliding or removal of nucleosomes from DNA (Flaus and Owen-Hughes 2011). A number of these chromatin remodeling complexes have been identified either biochemically or genetically as important for progression of the replication fork. ATP-utilizing chromatin assembly and remodeling factor (ACF) was initially discovered in *Drosophila* extracts and helps to reconstitute long arrays of regularly spaced nucleosomes in vitro (Ito et al. 1997; Varga-Weisz et al. 1997). One of the ACF subunits is ISWI, (imitation switch), an ATP-dependent chromatin remodeling protein found in a number of different complexes. Loss of another ACF subunit in *Drosophila*, ACF1, results in a more rapid progression through S phase and disrupts the polycomb-mediated silencing of facultative heterochromatin (Fyodorov et al. 2004). In mammalian systems ACF1 is required for the replication of pericentromeric heterochromatin (Collins et al. 2002). In vitro nucleosomal array reconstitution experiments indicate that ACF may facilitate the maturation of a nonnucleosomal histone octamer–DNA intermediate into a mature nucleosome (Torigoe et al. 2011). Another ISWI-containing complex, Williams syndrome transcription factor (WSTF), is targeted to the replication fork via an interaction with the DNA polymerase processivity factor PCNA to promote efficient DNA replication throughout S phase (Poot et al. 2004). The loss of WSTF results in an increased accumulation of the heterochromatin protein HP1 and increased chromatin compaction. In addition, WSTF is also important for the DNA damage response (Xiao et al. 2009). Together these data highlight how ATP-dependent chromatin remodelers can contribute to both the disruption of chromatin ahead of the forks as well as the reassembly of chromatin structure and maintenance of epigenetic states behind the fork.

Additional ATP-dependent chromatin remodeling complexes have been found to mediate histone dynamics at the replication fork in S phase. The INO80 complex and its catalytic component (SNF2) have been implicated in transcription, DNA damage repair, and fork progression during replicative stress (Bao and Shen 2007). INO80 also associates with stalled forks and early origins of replication, suggesting a possible role in initiation and replication fork restart (Shimada et al. 2008). In addition, INO80 and ISW2 can function in parallel to promote efficient fork progression through late-replicating regions of the *S. cerevisiae* genome (Vincent et al. 2008). It remains to be determined how these factors are targeted to the replication fork, as well as their relative contribution to chromatin disassembly ahead of the fork or reassembly behind the fork.

Histone chaperones are also likely involved in the disassembly of chromatin, where they may act as histone acceptors. A local concentration of parental histones may be maintained at the replication fork by the chaperone-mediated acceptance and sequestration of disassembled histones. Increasing evidence suggests that ASF1 and FACT can also act as histone acceptors at the replication fork (Groth et al. 2007; Jasencakova et al. 2010) for subsequent transfer to CAF-1 and NAP1 for incorporation into chromatin.

The H3–H4 chaperone, ASF1, is clearly linked to DNA replication, as is evident by the S-phase defects observed in a variety of organisms on ASF1 depletion (reviewed in Mousson et al. 2007), including replication fork defects and accumulation of cells in S phase (Groth et al. 2007). The function of ASF1 during DNA replication appears to be independent of CAF-1, as replication-coupled assembly is not impaired in chicken DT40 cells lacking ASF1 (Sanematsu et al. 2006). Recent experiments have shown that during replication stress ASF1 can associate with parental histones, suggesting a role as a histone acceptor (Groth et al. 2007; Jasencakova et al. 2010). In support of this idea, structural studies suggest that ASF1 binds the carboxyl terminus of H4 and that this “strand capture” can facilitate the splitting of the H3–H4 tetramer into dimers (English et al. 2006). Thus, a model begins to emerge of a histone

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chaperone "assembly line" at the replication fork, with ASF1 accepting parental H3–H4 tetramers, splitting them, and then passing them onto CAF-1 for deposition (Corpet et al. 2010). Importantly, defects in the recycling of parental histones could impact the transmission of parental PTMs, thereby compromising the stable maintenance of a given chromatin state.

An assembly line model for the disruption of nucleosomes and recycling of parental histones would require mechanisms to keep a population of histone chaperones at the replication fork. The FACT complex has been shown to interact with multiple fork components, including the Mcm2–7 helicase complex and replication protein A (RPA) (Gambus et al. 2006; Tan et al. 2006; VanDemark et al. 2006). Thus, FACT is well positioned to accept evicted H2A–H2B dimers and perhaps cooperate with NAP1 for their subsequent reassembly. Interestingly, organism-specific mechanisms appear to target ASF1 to the active replication fork. In *S. cerevisiae* ASF1 interacts with replication factor C (RFC), the PCNA clamp loader (Franco et al. 2005), whereas in mammalian cells ASF1 is connected to the Mcm2–7 helicase via an H3–H4 histone bridge (Groth et al. 2007). Together these results suggest a tight coupling between DNA unwinding at the replication fork and the disassembly and assembly of chromatin. Underscoring this connection, it has recently been shown in *S. cerevisiae* that the deposition of Okazaki fragments during lagging-strand synthesis occurs in nucleosomal-sized steps and is tightly coupled to assembly (Smith and Whitehouse 2012).

STOICHIOMETRY OF HISTONE DEPOSITION

The stability of the histone $(H3–H4)_2$ tetramer when it is free of DNA in solution (Baxevanis et al. 1991) led to the long-standing assumption that these histones were deposited directly as a tetramer. Recent data have now challenged this view. Specifically, histones H3 and H4 were found as dimers together with histone chaperones within predeposition complexes in human cells (Tagami et al. 2004). Moreover, the investigation of CenH3 (centromeric-specific H3) nucleosomes identified hemisomes containing one copy each of CenH3, H2A, H2B, and H4 (Dalal et al. 2007). Together these data suggest that histones H3–H4 or CenH3–H4 can be first provided as dimers. Two H3–H4 dimers then associate during deposition onto replicating DNA to form tetramers. In addition, the resolution of the crystal structure of ASF1 interacting with a dimer of histones H3–H4 revealed that ASF1 physically blocks the formation of an $(H3–H4)_2$ tetramer (English et al. 2006; Natsume et al. 2007). It is unclear whether both new histone H3–H4 dimers are provided by ASF1 and then deposited by CAF-1 onto DNA or whether additional chaperones are also involved. In vivo metabolic labeling studies led to the general view that ahead of the replication fork the core histone octamer is disrupted into two H2A–H2B dimers and a histone $(H3–H4)_2$ tetramer (Annunziato 2005). Recent studies using isotope labeling combined with mass spectrometry analysis of histone content have shed light on whether the histone $(H3–H4)_2$ tetramer remains intact during transfer (Xu et al. 2010). Although the vast majority of H3.1–H4 tetramers do not split, the investigators observed a significant number of splitting events for the H3.3-containing tetramers (Xu et al. 2010). Thus, although newly synthesized histones are provided as dimers (Tagami et al. 2004), three alternative modes for H3–H4 partitioning during nucleosome assembly can now be considered as real (Fig. 3). It will be important to determine whether mixing events are exclusively variant-specific or if they reflect particular histone dynamics associated with specific chromatin regions.

PTMs AND THE ESTABLISHMENT/ MAINTENANCE OF EPIGENETIC STATES

PTMs of histones are crucial for defining and maintaining the epigenetic state of chromatin (Kouzarides 2007). A diverse array of histone-modifying enzymes regulates the PTM of both newly synthesized nascent histones as well as parental nucleosomal histones. A consequence of the combinatorial nature of potential histone modifications is that the preexisting

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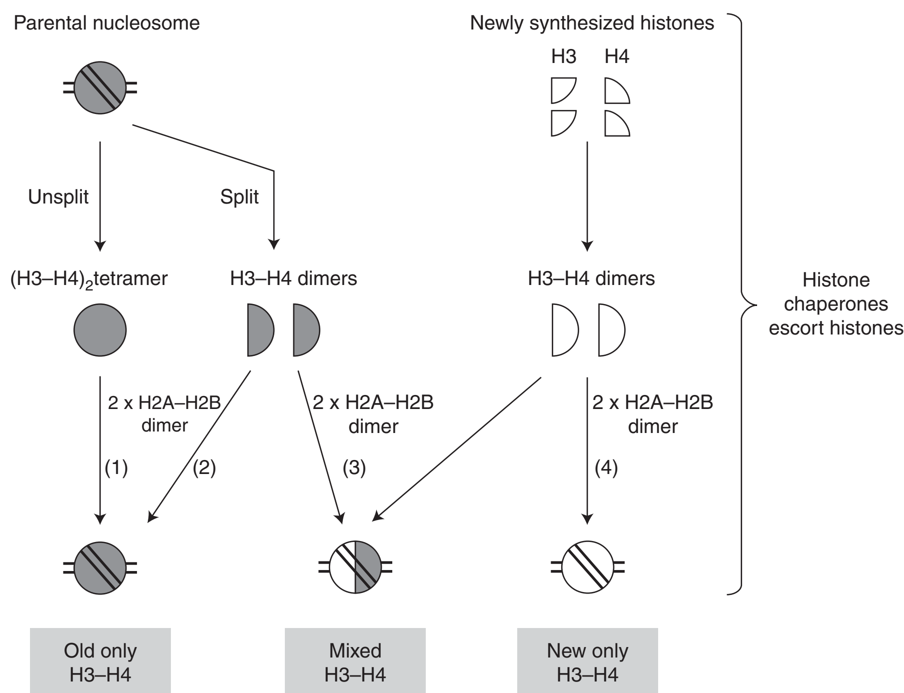

Figure 3. Partitioning of parental and newly synthesized histones. The deposition of newly synthesized histones or parental histones with existing PTMs can affect the inheritance and maintenance of specific chromatin states. Following disruption of nucleosomes at the replication fork, there are three possible outcomes for the deposition of parental and newly synthesized histones in the reassembled chromatin: deposition of parental H3–H4 only, deposition of mixed H3–H4 molecules composed of parental and nascent histones, or deposition of only newly synthesized H3–H4. On disassembly of the parental nucleosome, the $(H3–H4)_2$ tetramer can either remain intact (*1*) or split into two H3–H4 dimers (*2*). Deposition of the $(H3–H4)_2$ tetramer or deposition of two parental H3–H4 dimers followed by addition of two H2A–H2B dimers will result in the inheritance of a nucleosome with a parental H3–H4 tetramer core. Alternatively, the split H3–H4 dimers may associate with newly synthesized H3–H4 dimers (*3*), resulting in a nucleosome with a mixed H3–H4 tetramer core. Finally, the deposition of two newly synthesized H3–H4 dimers (*4*) will result in a nucleosome devoid of any parental histone PTMs.

modifications on a histone may dictate the subsequent action or inhibition of downstream histone-modifying enzymes. Thus, it is important to consider the initial PTMs of histones before deposition onto DNA to understand the specification and maintenance of specific chromatin states.

PTM of Newly Synthesized Histones

Almost all eukaryotic organisms show diacetylation of lysines 5 and 12 on newly synthesized histone H4 (Sobel et al. 1995; Loyola et al. 2006). The histone acetyltransferase (HAT) responsible for H4K5,K12ac is HAT1 (Parthun 2007), which forms a complex with the H3–H4 dimer, CAF-1, and ASF1 before deposition (Tagami et al. 2004; Loyola et al. 2006). The function of H4K5,K12ac remains an enigma, as the acetylation of H4K5,K12 is not required for histone deposition in vitro as unmodified H3–H4 histones are readily deposited on chromatin by CAF-1 in SV40 DNA replication assays (Shibahara et al. 2000) and in vivo (Ma et al. 1998). These results suggest that instead of facilitating chromatin deposition per se, the tight coupling of H4K5,K12ac with chromatin assembly may be important for marking a transient state (just having been replicated), which can impact the maintenance and propagation

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of heterochromatin if not removed (Taddei et al. 1999).

In *S. cerevisiae*, there is a peak of acetylation on lysine 56 on newly synthesized histone H3 that is observed during S phase (Kuo et al. 1996; Masumoto et al. 2005). H3K56 is located in the globular domain near the DNA entry/exit point of the nucleosome. Mutation of K56 to glutamine, commonly used as a constitutive acetylation mimic, results in an increased sensitivity to digestion by micrococcal nuclease (Masumoto et al. 2005), suggesting that H3K56ac weakens histone DNA interactions at the entry and exit points of the nucleosome. Consistent with the increased accessibility of nucleosomal DNA by H3K56ac, it promotes access to replication-coupled DNA damage to facilitate the repair of stalled replication forks and double-strand breaks (Driscoll et al. 2007; Han et al. 2007; Celic et al. 2008).

The acetylation of H3K56 in *S. cerevisiae* is mediated by the HAT Rtt109 in complex with either of two histone chaperones, ASF1 or Vps75 (a NAP1-related protein) (Han et al. 2007; Tsubota et al. 2007). The acetylation of H3K56 increases the affinity of CAF-1 for histone H3 and promotes the assembly of nucleosomes during S phase (Li et al. 2008). Thus, in the case of *S. cerevisiae*, there is a clear role for posttranslational histone modifications in promoting chromatin assembly. In contrast, the role of H3K56ac is less clear in mammals, as the abundance of the mark is very low (Garcia et al. 2007; Das et al. 2009; Xie et al. 2009) and not likely to be found on the bulk of newly synthesized histones. However, it remains formally possible that H3K56ac may be rapidly deacetylated following deposition. Further studies on the role of H3K56ac during replication-coupled chromatin assembly in mammals are clearly warranted.

The chromatin marks associated with nascent histones H3 and H4 are transient and rapidly removed during chromatin maturation as detected in heterochromatin regions (Taddei et al. 1999). The removal of H4K5 and K12 diacetylation is critical for the association of HP1 and the maintenance of silenced heterochromatin (Taddei et al. 1999), as well as proper centromere function (Ekwall et al. 1997; Taddei et al. 2001). Indeed, global defects in the removal of acetyl groups, mediated by the Cre recombinase-driven inactivation of histone deacetylase 3 (HDAC3), can lead to impaired S-phase progression and increased sensitivity to DNA damage (Bhaskara et al. 2008). Similarly, the deacetylation of H3K56 in *S. cerevisiae* during chromatin maturation is also important for genome stability. Loss of the two HDACs, Hst3 and Hst4, which target H3K56ac, leads to DNA damage (Celic et al. 2006) and sensitivity to replication stress (Celic et al. 2008). Thus, the proper maturation of chromatin is essential for genome stability.

In mammalian systems, newly synthesized histones are typically devoid of lysine methylation; however, it has recently been shown that there is a significant population of H3K9 monomethylation on predeposited H3.1 and H3.3 (Loyola et al. 2006). The histone methyltransferase SetDB1 targets H3K9 for monomethylation in complex with CAF-1 (Loyola et al. 2009). Deposition of nascent H3K9me may serve as a seed for the subsequent di- and trimethylation events mediated by the methyltransferase Suv39h, which recognizes the H3K9me1 specifically as its substrate (Loyola et al. 2009). In this manner, the mark incorporated before deposition may impact the final chromatin state. Of importance will be understanding the mechanisms of when and where a predeposition "seed" modification may be incorporated and the role of histone chaperones in specifying the deposition of the modified histone to influence chromatin state.

Epigenetics at the Replication Fork

DNA methylation and PTMs of histones are widely viewed to be conveyors of epigenetic information that is independent from the underlying genomic sequence. As the chromatin is disassembled on passage of the fork, there must be mechanisms to ensure the faithful reestablishment of epigenetic information following chromatin assembly. Finally, these mechanisms have to be plastic and able to dynamically respond to tissue-specific and developmental demands.

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DNA methylation of cytosine at CpG sequences is important for the maintenance of a repressive chromatin structure (Weber and Schubeler 2007). The symmetrical nature of CpG methylation implies that following DNA replication, each daughter molecule of DNA will be hemimethylated. The DNA methyltransferase Dnmt1 has a preferred affinity for hemimethylated CpG motifs (Pradhan et al. 1999) and can be targeted to the active replication fork via an interaction with PCNA, which suggests a straightforward model for the semiconservative inheritance of DNA methylation. However, it should be noted that the interaction between PCNA and Dmnt1 is not strictly required for the maintenance of DNA methylation following DNA replication (Schermelleh et al. 2007; Spada et al. 2007).

As with DNA methylation, PCNA serves as the common link between the replication fork and factors that propagate histone PTMs. For example, PCNA tethers CAF-1 to the fork (Shibahara and Stillman 1999; Moggs et al. 2000), and in addition to functioning as a histone chaperone, the p150 subunit of CAF-1 binds HP1 (Murzina et al. 1999) and promotes the redistribution of HP1 on chromatin during replication (Quivy et al. 2004). As the p150 subunit of CAF-1 is required for the replication of pericentric heterochromatin (Quivy et al. 2008), it may also function in the disassembly of chromatin and removal of HP1 ahead of the fork, followed by the subsequent deposition behind the fork of recycled parental histones presenting H3K9me3. HP1 serves to recruit the H3K9 methyltransferase Suv39h, thus establishing a positive-feedback loop to reestablish the pericentric heterochromatin state following passage of the replication fork. In addition, a number of other histone-modifying enzymes interact with PCNA, including the methyltransferase PR-Set7/Set8 (Jorgensen et al. 2007; Huen et al. 2008), as well as HDAC enzymes (Milutinovic et al. 2002), to ensure the local activity of these enzymes at the replication fork.

The maintenance of H3–H4 PTMs following DNA replication is complicated by the deposition of both parental and nascent histones behind the fork. The random model posits that parental $(H3–H4)_2$ with mature PTMs are mixed on either daughter strand with the newly synthesized histones. A consequence of this mixing is the dilution of the parental histone PTMs. On long arrays of nucleosomes, the relative local density of nucleosomes with parental histones relative to nascent histones could serve as a template to recruit histone-modifying enzymes to propagate the mark to the newly synthesized histones (Probst et al. 2009; Margueron and Reinberg 2010). This model is attractive for broad regions of similar chromatin states, such as the repressive H3K9m2/me3 marks found at pericentromeric heterochromatin. However, when one considers specific PTMs confined to only one or two nucleosomes, it becomes increasingly unlikely that the parental tetramer can serve as a template. However, the recent evidence of split H3–H4 dimers (Xu et al. 2010) may represent a mechanism to ensure that each daughter strand receives an H3–H4 dimer with the parental PTM. Thus, the modification would be propagated via an intraparticle mechanism. Other models (Probst et al. 2009) should also be considered, and it is possible that unique mechanisms for the propagation of chromatin states are associated with specific loci and particular developmental stages.

NUCLEOSOME POSITIONING

In the wake of the replication fork, chromatin is assembled into nucleosomes that are often deposited in specific locations relative to functional regulatory elements. This nucleosome positioning can be maintained between cell divisions and throughout the population. The location and occupancy of nucleosomes throughout the genome partly governs the accessibility of the DNA for binding by *trans*-acting factors. A series of classic experiments at the *PHO5* locus in *S. cerevisiae* highlighted the importance of nucleosome occupancy in modulating the transcriptional response and the binding of *trans*-acting factors (Almer and Horz 1986; Almer et al. 1986; Raser and O'Shea 2004). The regulatory consequence of nucleosome positioning

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also impacts other nuclear processes including DNA recombination (Roth and Roth 2000) and replication (Simpson 1990; Lipford and Bell 2001; Eaton et al. 2010).

The advent of genomic technologies has made it possible to systematically survey nucleosome positioning throughout eukaryotic genomes (for review, see Radman-Livaja and Rando 2010). Briefly, chromatin is digested by micrococcal nuclease, mononucleosomes are isolated, and the 147-bp DNA fragments are recovered. Genomic tiling microarrays or next-generation sequencing is used to map the short DNA fragments back to the genome and determine nucleosome occupancy. The locations of nucleosomes relative to annotated chromosomal features, such as transcription start sites (TSSs), are remarkably similar across different eukaryotic organisms (Yuan et al. 2005; Whitehouse et al. 2007; Mavrich et al. 2008b; Schones et al. 2008; Valouev et al. 2008). Typically, an array of well-positioned nucleosomes starting from the TSS and progressing into the gene body is observed, with a region of low nucleosome occupancy immediately upstream of the TSS. This nucleosome-depleted region is most pronounced at actively transcribed genes and is often enriched for *cis*-acting regulatory elements and *trans*-acting factors.

Nucleosome positioning throughout the genome is mediated by both *cis*- and *trans*-acting factors. The histone octamer core does not bind specific sequences through a recognizable DNA-binding domain. Rather, flexibility constraints of the DNA, imposed by the necessity of wrapping the DNA almost two times around the histone core, specify preferential sequences. For example, dinucleotides of AA, TT, or TA at 10-bp intervals, by favoring a proper DNA curvature, increased affinity for the histone octamer (Anselmi et al. 1999; Thastrom et al. 1999). In contrast, poly(A)-rich tracts are relatively inflexible and do not favor nucleosome occupancy (Iyer and Struhl 1995). Analysis of the DNA sequence properties from the in vivo nucleosome positioning studies has led to the proposal of a nucleosome positioning code (Segal et al. 2006)—that the underlying sequence is predictive of nucleosome positioning throughout the genome. This nucleosome positioning code was tested in part by depositing reconstituted chicken histone octamers onto yeast DNA in vitro in the absence of any *trans*-acting factors such as transcription factors or chromatin remodeling activities (Kaplan et al. 2010). Indeed, the reconstituted nucleosomes were highly correlated (0.74) with the in vivo nucleosome positioning data, although they did not capture all of the in vivo nuances. Although specific sequences do contribute to the exclusion and positioning of nucleosomes, *trans*-acting factors also have an important role in specifying nucleosome position. *Trans*-acting factors may act as a fixed barrier, with the nucleosomes nearest the barrier showing the highest degree of positioning, followed by a gradual loss of positioning with increasing distance from the barrier (Mavrich et al. 2008a). Finally, it will be important to understand how initial histone deposition and chromatin assembly are coordinated with the reassociation of *trans*-acting factors behind the replication fork to reestablish nucleosome positioning for proper genome function.

CHROMATIN AND THE REGULATION OF REPLICATION ORIGINS

The selection and activation of DNA replication origins must occur within the context of the local chromatin environment. Early studies examining the patterns of radiolabeled thymidine incorporation found that specific chromosomal domains were replicated at discrete times in S phase (Goldman et al. 1984). Transcriptionally active euchromatin replicated early in S phase, whereas the condensed and gene-poor heterochromatin was copied at the end of S phase. These and similar experiments (Stambrook and Flickinger 1970) suggested that the local chromatin environment affected not only the transcription program but also the DNA replication program.

In *S. cerevisiae*, origins of DNA replication were first identified as short autonomously replicating sequence (ARS) elements that were required to propagate and maintain an episome. Each ARS is defined, in part, by a degenerate *cis*-acting sequence element termed the ARS

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consensus sequence (ACS). The ACS is necessary but not sufficient for origin function (Celniker et al. 1984), as there are >10,000 matches to the ACS motif in the yeast genome (Breier et al. 2004). However, <400 of those potential ACS matches function as bona fide binding sites for the origin-recognition complex (ORC) (Xu et al. 2006). Thus, other chromosomal features are likely involved in defining replication origins.

Despite the conservation of ORC and other *trans*-acting factors required for the assembly of the prereplicative complex (pre-RC) and loading of the Mcm2–7 helicase at origins, no conserved *cis*-acting elements directing DNA replication have been identified in higher eukaryotes (Gilbert 2004). Unlike in *S. cerevisiae*, ORC purified from higher eukaryotes shows little or no sequence specificity in vitro (Vashee et al. 2003; Remus et al. 2004). Together these results might suggest that origin selection and replication initiation are random or stochastic events in higher eukaryotes, but numerous studies have identified specific origins of replication as well as ORC binding at specific and reproducible chromosomal locations (Austin et al. 1999; Ladenburger et al. 2002; Karnani et al. 2010; MacAlpine et al. 2010). Thus, in contrast to the sequence cues that contribute to origin selection in yeast, the determinants of ORC binding in higher eukaryotes appear to be primarily dependent on the local chromatin environment.

Together these results suggest that in both lower and higher eukaryotes the local chromatin organization and structure are important features of origin selection. Indeed, recent studies in multiple experimental model systems have highlighted the role of histone modifications and chromatin organization in regulating the DNA replication program.

Histone Modifications and Origin Regulation

Experiments in *S. cerevisiae* showed the importance of the local chromatin environment in regulating origin function. In yeasts, the genes near the ends of the chromosome are often silenced by Sir2p, an HDAC, and are typically late-replicating (reviewed in Rusche et al. 2003). The delay in origin activation near the telomeres is not caused by sequence, as transposition of ARS501, a telomeric late-activating origin, to another region of the genome relieved the telomeric suppression of origin activation (Ferguson and Fangman 1992). Instead, the repression of origin activation near the telomere is because of the local chromatin structure, and disruption of that structure led to an advancement of origin firing earlier in S phase (Stevenson and Gottschling 1999). More recently, the genome-wide analysis of the time at which specific sequences are replicated in S phase has identified a clear correlation with transcriptional activity and replication timing in human (Birney et al. 2007; Ryba et al. 2010), mouse (Hiratani et al. 2010), *Drosophila* (Schübeler et al. 2002; MacAlpine et al. 2004), and chicken cells (Hassan-Zadeh et al. 2012). Gene-dense, transcriptionally active regions of the genome are replicated before regions of the genome with sparse gene activity. The correlation between replication timing and transcriptional activity is not at the level of individual genes but rather is defined by the broad transcriptional activity of large chromosomal domains (MacAlpine et al. 2004). Not surprisingly, there is also a strong correlation between chromatin modifications associated with active transcription and early replication. Experiments from the encyclopedia of DNA elements (ENCODE) project found that sequences replicated in early S phase from HeLa cells were enriched for activating chromatin marks (H3K4Me2 and H3K4Me2) as well as the hyperacetylation of histones H3 and H4 (Birney et al. 2007). Similar broad correlations between time of replication and activating histone modifications have been reported in numerous independent studies from a number of eukaryotic model systems (Hiratani et al. 2008; Schwaiger et al. 2009; Lee et al. 2010). The apparent coordination between the transcription and replication programs may, in part, be caused by the frequent localization of replication origins near the start sites of transcription (Cadoret et al. 2008; Cayrou et al. 2011).

Perturbation of histone acetylation impacts the replication program. In the absence

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of Rpd3, an HDAC, the length of S phase is shortened, presumably because of the earlier activation of a subset of replication origins (Vogelauer et al. 2002). Indeed, genome-wide analysis of origin activity in the absence of Rpd3 resulted in the earlier activation of almost 100 late-firing origins of replication (Knott et al. 2009). A local increase in histone acetylation mediated by tethering Gcn5, a histone acetyltransferase, to a late-activating origin also promoted origin initiation significantly earlier in S phase (Vogelauer et al. 2002). The role of histone acetylation in promoting replication is not specific to yeast, as the recruitment of Chameau (Hbo1), a *Drosophila* histone acetyltransferase (see below), to the chorion locus stimulated replication activity (Aggarwal and Calvi 2004). Similarly, the targeting of a HAT or HDAC activity to the B-globin locus can shift the time of replication from late to early and vice versa, respectively (Goren et al. 2008). These results clearly indicate that local changes in histone acetylation are able to fine-tune origin activity.

The recent finding that the Orc1 bromo-adjacent homology (BAH) domain specifically binds dimethylated lysine 20 of histone H4 (Kuo et al. 2012) suggests that H4K20me2 may be important for ORC recruitment to chromatin and defining replication origins. Mutations in the Orc1 BAH domain as well as other pre-RC components result in Meier–Gorlin syndrome, a rare primordial form of dwarfism (Bicknell et al. 2011). Furthermore, the loss of Suv4-20h1/h2, the methyltransferase responsible for H4K20me2, results in similar developmental defects to Meier–Gorlin syndrome in zebrafish, suggesting a critical role for H4K20 dimethylation and the DNA replication program during normal development (Kuo et al. 2012). However, it should be noted that H4K20me2 is the most abundant histone modification, accounting for >85% of histone H4 levels in the mouse (Schotta et al. 2008). On average, 97% of all nucleosomes will contain at least one histone H4K20me2, which makes it hard to argue that H4K20me2 is a specificity factor for ORC. Instead, H4K20me2 may simply help to stabilize ORC on chromatin, with other chromosomal features acting as origin specificity factors.

Instead of influencing ORC localization, new experiments suggest that the local chromatin environment may regulate the loading of the replicative helicase in G_1. Hbo1 (histone acetylase binding to ORC) is an abundant histone acetyltransferase responsible for the bulk of histone H4 acetylation in mammalian genomes and was initially identified based on its interaction with ORC, Mcm2, and Cdt1 (Iizuka and Stillman 1999; Burke et al. 2001; Iizuka et al. 2006). In *Xenopus* extracts Hbo1 is required for pre-RC assembly (Iizuka et al. 2006), and the artificial recruitment of catalytically nonfunctional Hbo1 to a mammalian origin of replication negatively impacts the loading of the Mcm2–7 helicase (Miotto and Struhl 2010). Interestingly, like many proteins with HAT activity, Hbo1 has the ability to acetylate nonhistone proteins including Orc2, Mcm2, and Cdc6 in vitro (Burke et al. 2001). Thus, it is not entirely clear if the role of Hbo1 in regulating origins is because of the specific acetylation of histones or, alternatively, the acetylation of pre-RC components.

Similar experiments also suggest that the levels of H4K20 monomethylation are important for helicase loading and pre-RC formation (Tardat et al. 2007). Set8, also known as PR-Set7, is a cell-cycle-regulated methyltransferase that monomethylates histone H4 at lysine 20 (Fang et al. 2002; Nishioka et al. 2002). Set8 is targeted to the proteasome during S phase by the E3 ubiquitin ligase Crl4 in a PCNA-dependent manner (Abbas et al. 2010; Centore et al. 2010; Oda et al. 2010). Set8 levels are critical for maintaining genomic stability, as the loss of Set8 function results in an S-phase delay, chromosome decondensation, increased DNA damage, G_2 arrest, and centrosome amplification (Karachentsev et al. 2005; Jorgensen et al. 2007). Similarly, the stabilization and overexpression of Set8 is also detrimental to the cell, resulting in premature chromatin compaction and rereplication (Tardat et al. 2007). Presumably, the rereplication induced by Set8 stabilization is due in part to promiscuous pre-RC formation, as a local increase in H4K20me and the recruitment

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of pre-RC components are observed when Set8 is tethered to a specific locus (Tardat et al. 2007). Understanding how the methylation state of lysine 20 (mono-, di-, or trimethylation) is regulated through the cell cycle and its impact on ORC localization, pre-RC assembly, and genome stability will undoubtedly be an active and important area of future research.

Nucleosome Positioning and Origin Selection

Early experiments on an episome containing ARS1, an *S. cerevisiae* origin of DNA replication, identified well-positioned nucleosomes flanking the origin sequence (Simpson 1990). The analysis of genome-wide nucleosome positioning data sets from *S. cerevisiae* revealed that almost all ARS elements are depleted of bulk nucleosomes (Mavrich et al. 2008a). Subsequent analysis of nucleosome positioning relative to the orientation of the 17-bp ACS, and not the broadly mapped ARS elements, identified precisely positioned nucleosomes flanking almost all yeast origins of replication (Berbenetz et al. 2010; Eaton et al. 2010). Thus, the positioned nucleosomes first observed at ARS1 on a plasmid appear to be a defining feature of *S. cerevisiae* origins of DNA replication. Furthermore, decreased nucleosome occupancy at origins of replication has also been observed in a variety of different eukaryotic organisms outside of *S. cerevisiae*, including *Drosophila* (MacAlpine et al. 2010), mammalian cells (Lubelsky et al. 2011), and *Schizosaccharomyces pombe* (Givens et al. 2012).

An emerging model is that in *S. cerevisiae*, the ACS and downstream sequence cues keep the origin region free from encroaching nucleosomes and that this large nucleosome-free region facilitates ORC localization (Fig. 4). Once bound, ORC and presumably an ATP-dependent chromatin remodeling activity are required to generate the array of well-positioned nucleosomes flanking the origin of replication (Eaton et al. 2010). Interestingly, if the positioning of the upstream nucleosome is altered, Mcm2–7 loading and initiation are impaired, suggesting that the chromatin architecture is critical for pre-RC assembly and origin

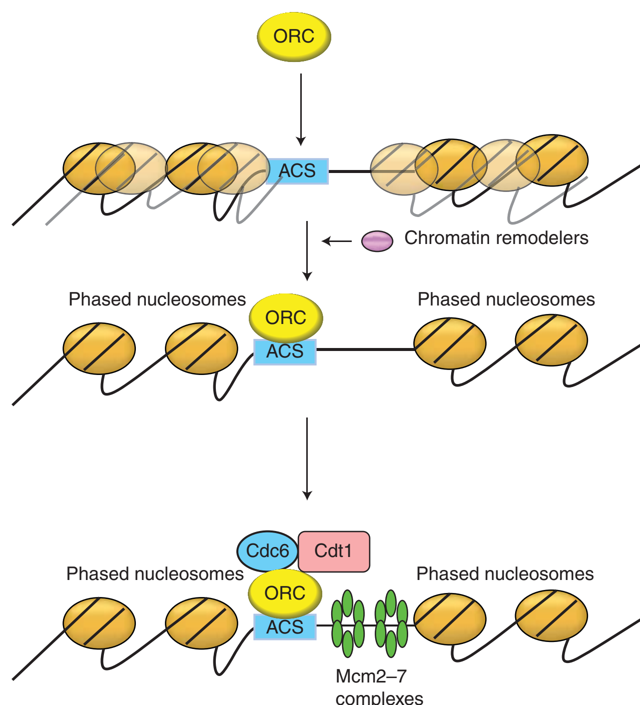

Figure 4. Nucleosome organization at origins of replication. The AT-rich nature of the ACS and flanking sequences at *S. cerevisiae* origins of replication prevent encroachment of nucleosomes into the origin. The nucleosome-free region at origins of replication is observed in yeast and higher eukaryotes and may function as a primary determinant for ORC binding. On ORC binding the flanking nucleosomes become precisely positioned, and this positioning is dependent on ORC and an ATP-dependent chromatin remodeling activity. The nucleosome-free region at the origin may facilitate the loading of multiple Mcm2–7 complexes and subsequent DNA unwinding events before initiation.

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activation (Lipford and Bell 2001). However, several important questions remain. How do nucleosome positioning, turnover, and eviction facilitate Mcm2–7 loading and origin activation? Which ATP-dependent chromatin remodeling activities are required, and how do they contribute to origin activation, timing, and efficiency? Understanding how the local chromatin structure impacts and regulates the relatively well-defined *S. cerevisiae* DNA replication program will be critical for understanding how the local chromatin environment and dynamics contribute to the plasticity of origin selection in higher eukaryotes.

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